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Oxidative damages induced by short-term exposure to cadmium in bean plants: Protective role of salicylic acid

Issam Saidi [a,*], Marwa Ayouni [a], Amina Dhieb [b], Yassine Chtourou [b], Wided Chaïbi [a], Wahbi Djebali [a]

[a] Unité de Recherche de Biologie et Physiologie Cellulaires Végétales, Faculté des Sciences de Tunis, Campus Universitaire, 1060 Tunis, Tunisia
[b] Laboratoire de Biotechnologie, Faculté des Sciences de Sfax, Route Sokra, B.P. 1171, 3000 Sfax, Tunisia

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ABSTRACT

The role of salicylic acid (SA) in alleviating cadmium (Cd) toxicity was investigated in a hydroponic cultivation system. Short-term exposure of bean (*Phaseolus vulgaris*) plants to 20 μM Cd inhibited biomass production and intensively increased accumulation of Cd in both roots and leaves. At leaf level, Cd significantly decreased mineral ions, chlorophyll and carotenoids concentrations. Concomitantly, Cd enhanced electrolyte leakage, H_2O_2 content and lipid peroxidation as indicated by malondialdehyde (MDA) accumulation. SA pretreatment decreased the uptake and the transport of Cd, alleviated the Cd-induced inhibition of nutrient absorption and led to a significant increase of chlorophyll and carotenoid content. SA application alleviated the oxidative damages as evidenced by the lowered H_2O_2 and MDA content. SA particularly induced an increase in both CAT and APX activities accompanied by a significant reduction in SOD and POD activities. As important antioxidants, ascorbate and glutathione contents in bean leaves exposed to cadmium were significantly decreased by SA treatment. These results reveal the potentiating effect of salicylic acid in regulating cadmium induced oxidative stress in bean plants.

1. Introduction

Cadmium (Cd) is an important environmental pollutant and a potent toxicant to organisms, including plants (Harminder et al., 2008). Its toxic symptoms in plants can be linked to changes in morphological, physiological and biochemical processes, leading to growth perturbation and cell death (Seregin and Ivanov, 2001).

At the morphological level, an excessive amount of Cd causes stunted growth, root browning, leaf epinasty, chlorosis and necroses (Benavides et al., 2005). At cell level, intoxication by Cd induces oxidative stress as evidenced by enhanced lipid peroxidation, hydrogen peroxide (H_2O_2) generation and ion leakage (Smeets et al., 2005). Cd can enhance prooxidant status by decreasing the antioxidant glutathione (GSH) pool, activating calcium-dependent systems and affecting iron-mediated processes (Pinto et al., 2003). It can also disrupt photosynthetic electron chain, leading to oxygen radical production (Asada and Takahashi, 1987). Cd toxicity may result from the binding of metal to sulphydryl groups in proteins, leading to inhibition of activity or disruption of structure (Benavides et al., 2005; Romero-Puertas et al., 2004). Metal interaction with ligand groups of enzymes largely defines its toxicity and the inhibition of enzymes may be due to masking of catalytically-active groups (Das et al., 1997). To survive against Cd toxicity, the plants have evolved several protective mechanisms. One of them is the response of antioxidant enzymatic system, which involves the sequential and simultaneous action of a number of enzymes such as SOD, CAT, POD and APX and non-enzymatic scavengers such as glutathione (GSH), ascorbate (AsA), carotenoids (Cars) and α-tocopherol, that are responsible for scavenging excessively-accumulated reactive oxygen species (ROS) in plants under stress conditions (Shah et al., 2001). Among these defenses, SODs are a group of enzymes that accelerate the conversion of superoxide radicals to H_2O_2 (Fernández-Ocaña et al., 2011). CAT is one of the main H_2O_2-scavenging enzymes that dismutates H_2O_2 into H_2O and O_2 (Corpas et al., 1999). PODs are enzymes that catalyze the H_2O_2-dependent oxidation of a wide variety of substrates, mainly phenolics (Kawano, 2003). GSH, a disulfide reducer, plays a central role in protecting plants from ROS. It protects thiols of enzymes, regenerates AsA and reacts with singlet oxygen, H_2O_2 and hydroxyl radicals (Foyer, 1993). AsA is an important antioxidant that reacts not only with H_2O_2, but also with O_2^-, OH^- and lipid hydroperoxides (Ramachandra et al., 2004). Depending on metal concentrations, Cd can either inhibit or stimulate the activities of these antioxidants before visible symptoms of toxicity appear (Correa et al., 2006).

Salicylic acid (SA), a naturally occurring plant hormone, influences various physiological and biochemical functions in plants, acts as an important signaling molecule and has diverse effects on tolerance to

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; Cars, carotenoids; CAT, catalase; Chl, chlorophyll; GSH, glutathione; MDA, malondialdehyde; POD, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author at: Département de Biologie, Unité de Recherche de Biologie et Physiologie Cellulaires, Végétales, Faculté des Sciences de Tunis, Campus Universitaire, 1060 Tunis, Tunisia. Tel.: +216 22469321.
E-mail address: issambenahmed@yahoo.fr (I. Saidi).

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biotic stress (Horvath et al., 2007). Its role in plant tolerance to abiotic stresses such as drought (Munne-Bosch and Penuelas, 2003), chilling (Janda et al., 1999; Kang and Saltveit, 2002), heavy metals (Belkhadi et al., 2010), heat (Larkindale and Knight, 2002) and osmotic stress (Borsani et al., 2001) has also been reported. In addition, SA could contribute to maintaining cellular redox homeostasis through the regulation of antioxidant enzyme activity (Slaymaker et al., 2002) and induction of the alternative respiratory pathway (Moore et al., 2002) as well as to regulating gene expression by inducing an RNA-dependent RNA polymerase that is important for post transcriptional gene silencing (Xie et al., 2001). Therefore the present study was undertaken to determine the physiological and biochemical changes in bean plant pretreated with SA during Cd-induced stress and to investigate the possible mediatory role of SA in protecting plants from Cd-induced oxidative stress.

2. Material and methods

2.1. Plant material and growth conditions

Bean seeds (*Phaseolus vulgaris*) were disinfected with 1% NaOCl for 5 min, then washed thoroughly with distilled water and germinated between wet paper towels at 24 °C in the dark. Four days after, obtained seedlings were transferred into plastic beakers (6 L capacity, 6 plants per beaker) filled with nutrient solution containing: 1.0 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2$, 1.0 mM KH_2PO_4, 2.0 mM KNO_3, 2.0 mM NH_4Cl, 50 μM EDTA–Fe–K, 30 μM H_3BO_3, 10 μM MnSO4, 1.0 μM $ZnSO_4$, 1.0 μM $CuSO_4$ and 30 μM $(NH4)_6Mo7O_{24}$. After an initial growth period of 7 days in different SA concentrations (10, 50 and 100 μM), treatments were performed by adding 20 μM $CdCl_2$ to the nutrient solution. Hence, eight treatments of five replicates each were established, including a control (neither SA-pretreated nor Cd-stressed), Cd-stressed only, SA-treated only and SA–Cd-stressed (pretreatment with SA and then Cd-stressed). Plants were grown in a growth chamber with a 16-hour photoperiod and a 25 °C/20 °C regime, irradiance of 150 μmol m^{-2} s^{-1} and 65–75% relative humidity. The nutrient solution was buffered to pH 5.5 with HCl/KOH, aerated and changed twice per week. After 3 days of Cd-treatment, all plant organs were harvested, thoroughly washed with water, soaked in 20 mM EDTA for 15 min to remove adsorbed metals on the root surfaces and rinsed with distilled water. For biochemical analyses, primary leaves were harvested and immediately stored in nitrogen liquid. In each treatment group, five plants were examined for biochemical analysis.

2.2. Plant growth parameters and water absorption capacity (WAC)

Samples were oven-dried at 90 °C for 15 min, kept at 70 °C for 24 h to obtain a constant weight and weighted for dry biomass. Root and leaf water absorption capacity (WAC) was calculated using the following formula;

$$\text{Water Absorption Capacity } (\%) = 100 \times (\text{FW} - \text{DW})/\text{FW}$$

where FW and DW are fresh weight and dry weight of the plant materials, respectively.

2.3. Determination of ion concentrations

Dry plant material was powdered and wet-digested in acid mixture (HNO_3:$HClO_4$, 3:1, v/v) at 100 °C. Ion concentrations were estimated by atomic absorption spectrophotometry (Perkin-Elmer, AAnalyst 300) using an air–acetylene flame. The translocation factor (TF) was calculated as TF = 100 × [Cd] leaf/[Cd] root (Ait et al., 2002).

2.4. Determination of chlorophyll and carotenoid concentrations

Leaf chlorophyll was extracted from 100 mg FW with 80% chilled acetone and estimated by the method of Arnon (1949). Carotenoid concentration in the same extract was calculated by the formula given by McKinney (1941).

2.5. Determination of antioxidative enzyme activities

Frozen leaf tissue (0.4 g) was homogenized in 4 mL ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0, 4% PVP 40) using a pre-chilled mortar and pestle. The homogenate was squeezed through a nylon mesh and centrifuged for 30 min at 14,000 *g* at 4 °C. The supernatant was used for assays of the activities of SOD, CAT, POD and APX. All spectrophotometric analyses were conducted at 25 °C.

The activity of SOD (EC1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Beauchamp and Fridovich (1971). The reaction mixture (1 mL) included 50 mM phosphate buffer (pH 7.4), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 100 μL enzyme extract. The reaction was allowed to proceed for 15 min illuminated with fluorescent tubes. Absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT. SOD activity was expressed as U mg^{-1} protein.

CAT (EC1.11.1.6) activity was assayed by the decomposition of hydrogen peroxide according to Aebi (1984). The reaction mixture (1 mL) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.1% H_2O_2 and 100 μL enzyme extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient ($E = 39.4$ mM^{-1} cm^{-1}). CAT activity was expressed as μmol H_2O_2 min^{-1} mg^{-1} protein.

The activity of POD (EC1.11.1.7) was determined in terms of oxidation of guaiacol by measuring increase in absorbance at 470 nm (Chance and Maehly, 1955). The reaction solution (1 mL) was composed of 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 1.0 mM H_2O_2 and 0.1 mM EDTA and 100 μL enzyme extract. The reaction was started by addition of H_2O_2 and the increase in absorbance was recorded at 470 nm ($E = 26.6$ mM^{-1} cm^{-1}). POD activity was expressed as U mg^{-1} protein.

APX (EC1.11.1.1) activity was determined by the method of Nakano and Asada (1981). The reaction mixture (2 mL) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.3 mM ascorbate, 0.1 mM H_2O_2 and 100 μL enzyme extract. The reaction was initiated by addition of H_2O_2 and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm. Activity of APX was calculated by using the molar extinction coefficient for ascorbate ($E = 2.8$ mM^{-1} cm^{-1}). Activity was expressed as U mg^{-1} protein.

2.6. Determination of antioxidative compound concentrations

Glutathione (GSH) concentration was determined by the method of Ellman (1959) based on the development of a yellow color when 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was added to compounds containing sulfhydryl groups. 500 μL tissue homogenate in phosphate buffer was added to 3 mL 4% (v/v) sulfosalicylic acid. The mixture was centrifuged at 3000 *g* for 15 min. Then, 500 μL supernatant were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min of reaction. Total GSH concentration was expressed as μmol g^{-1}.

Ascorbate (AsA) concentration was determined spectrophotometrically by using dinitrophenyl-hydrazine according to Mukherjee and Choudhuri (1983). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA. Briefly, leaf samples were powdered in liquid nitrogen and extracted in 6% (w/v) trichloroacetic acid (TCA), 2% (w/v)

dinitrophenyl-hydrazine in 50% H_2SO_4 and 10% (v/v) thiourea in 70% ethanol. The homogenate was boiled in a water bath for 15 min, cooled at room temperature, and centrifuged at 1000 *g* for 10 min at 4 °C. The resulting pellet was dissolved with 80% H_2SO_4. The absorbance was measured at 530 nm. A calibration curve was prepared using ascorbic acid as standard and utilized for calculations. Results were expressed as μmol g^{-1} FW.

2.7. Determination of hydrogen peroxide concentration

Hydrogen peroxide (H_2O_2) levels were determined according to Sergiev et al. (1997). Leaf tissue (0.5 g) was homogenized in an ice bath with 5 mL 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 *g* for 15 min and 0.5 mL of the supernatant was added to 0.5 mL potassium phosphate buffer (10 mM, pH 7.0) and 1 mL potassium iodide (KI) (1 M). The absorbance of the supernatant was measured at 390 nm, the concentration of H_2O_2 was obtained using a standard curve. Results were expressed as nmol g^{-1} FW.

2.8. Determination of lipid peroxidation and membrane permeability

The level of lipid peroxidation in plant leaves was determined by estimation of the thiobarbituric acid reactive substances which was expressed as the malondialdehyde (MDA) concentration based on the method of Hodges et al. (1999). Briefly, fresh leaf sample (0.2 g) was ground in 0.1% (w/v) TCA and the homogenate was centrifuged at 10,000 *g* for 5 min. To 1 mL supernatant, 4 mL thiobarbituric acid (TBA) [5% TBA (w/v) in 20% trichloroacetic acid (w/v)] was added. The mixture was heated at 100 °C for 30 min and then cooled in an ice bath. After centrifugation at 10,000 *g* for 10 min, the absorbance of the supernatant was measured at 532 nm. The value was corrected for the non specific absorption at 600 nm. Lipid peroxidation level was expressed as nmol MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}. Membrane permeability was determined as follows. Fresh leaf sample (100 mg) was stirred for 30 min in deionised water followed by measurement of bathing medium conductivity (EC1). Then, the sample was boiled for 15 min and the final conductivity (EC2) of the medium was measured. Electrolyte leakage (%) was calculated using the formula: $(EC1/EC2)\times 100$.

2.9. Determination of soluble protein concentration

Soluble protein concentration was measured according to Bradford (1976) using the BCA protein assay reagent (Pierce, BCA Protein Assay Kit, USA) with bovine serum albumin (BSA) as the standard protein.

2.10. Statistical analysis

The experimental design was randomized with eight treatments and five replicates per treatment. The experiment was repeated three times under the same conditions. All statistical analyses were carried out with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA). Significant differences between treatment effects were determined by 1-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons with statistical significance of $P<0.05$.

3. Results

3.1. Effects of SA pretreatment on seedling growth under Cd stress

Under Cd treatment (20 μM), plant growth was negatively affected, reducing root and leaf dry weights by about 53.0% and 59.1%, respectively (Table 1). Moreover, the applied Cd concentration noticeably increased (15%) Root/Shoot ratio (Table 1). This indicates that leaves were more affected by Cd treatment than roots. However, under Cd stress conditions, seedlings pretreated with different concentrations of SA grew better than plants grown without SA application. Although SA pretreatments did not affect root dry weights of plants grown with or without Cd, they markedly alleviated Cd-induced leaf growth inhibition. At 10, 50 and 100 μM SA, leaf dry weights were respectively 60.4%, 89.5% and 77.8%, higher than in Cd-stressed plants grown without SA pretreatment. The SA pretreatment by itself increased leaf dry weights in a dose-dependent manner (Table 1). Moreover, as a result of SA application, the Root/Shoot ratio significantly decreased in comparison with that of plants grown at the presence of Cd without SA pretreatment. This suggests that SA stimulated shoot growth more than root growth. However, the used Cd and SA concentrations did not lead to significant changes of water balance (Table 1).

Table 1
Effects of SA pretreatment and/or 3-day Cd treatment (20 μM) on biomass accumulation and water absorption capacity (WAC) in 14-day old bean seedlings. Abbreviations: RWAC: Root Water Absorption Capacity; and LWAC: Leaf Water Absorption Capacity. Means of $n=5\pm SE$ from three independent experiments. Different letters mean significance of difference between the treatments ($P<0.05$, Tukey's test).

SA (μM)	Cd in nutrient solution	
	0 (μM)	20 (μM)
Root DW (mg plant^{-1})		
0	135.62 ± 0.015a	63.72 ± 0.020b
10	130.14 ± 0.051a	69.57 ± 0.025b
50	131.73 ± 0.082a	67.47 ± 0.030b
100	129.77 ± 0.039a	71.17 ± 0.024b
Leaf DW (mg plant^{-1})		
0	180.05 ± 0.041a	73.61 ± 0.0260c
10	203.22 ± 0.010ab	118.08 ± 0.041 cd
50	234.26 ± 0.007b	139.52 ± 0.070d
100	226.03 ± 0.012b	130.94 ± 0.052d
RWAC (%)		
0	89.42 ± 0.386a	84.87 ± 0.159a
10	88.67 ± 0.284a	90.00 ± 0.368a
50	89.10 ± 0.458a	90.52 ± 0.381a
100	89.01 ± 0.534a	89.38 ± 0.316a
LWAC (%)		
0	89.88 ± 0.111a	90.56 ± 0.233a
10	89.92 ± 0.587a	90.14 ± 0.402a
50	90.77 ± 0.109a	90.15 ± 0.026a
100	90.49 ± 0.424a	90.26 ± 0.131a

3.2. Effects of SA pretreatment on Cd accumulation

Cadmium addition to the nutrient solution resulted in a high Cd accumulation within roots, reaching 3021 μg g^{-1} DW, a value that is 55 times higher than that of shoots (Table 2). Pretreatment with SA before application of Cd significantly decreased Cd concentration in both roots and leaves (Table 2). In comparison with Cd treatment, pretreatment with SA (100 μM) decreased Cd concentration by 58.6%

Table 2
Effects of SA pretreatment on Cd concentrations (μg g^{-1} DW) in roots and leaves of 14-d-old bean plants submitted during 3 days to 20 μM $CdCl_2$. Abbreviations: TF, translocation factor. Means of $n=5\pm SE$ from three independent experiments. Different letters mean significance of difference between the treatments ($P<0.05$, Tukey's test).

SA (μM)	Cd concentration (μg g^{-1} DW)		TF (%)
	Roots	Leaves	
0	3021.3 ± 0.050a	55.12 ± 0.047a	1.82 ± 0.021a
10	2012.1 ± 0.152b	22.50 ± 0.093c	1.11 ± 0.111c
50	1770.5 ± 0.090b	24.00 ± 0.017c	1.35 ± 0.012b
100	1251.2 ± 0.106c	21.30 ± 0.087c	1.70 ± 0.015a

and 61.4%, respectively in roots and leaves. However, the translocation factor (TF) of Cd from roots to shoots decreased only with low SA pretreatments (10 and 50 μM) (Table 2). Moreover, under the influence of SA, there occurred an inhibition of Cd accumulation in whole seedlings in a dose-dependent way (Table 3).

3.3. Effects of SA and Cd on the nutrient concentrations

Changes in leaf K, Ca, Mg and Fe concentrations are presented in Table 4. As applied separately, Cd induced a significant decrease in K, Ca, Mg and Fe concentrations. SA alone increased significantly K concentration, but showed no significant effect on Ca, Mg, and Fe concentrations in comparison with the control. Pretreatment with 10, 50 and 100 μM SA before exposure to Cd increased K concentration by respectively 39.6%, 40.3% and 38.5% increase in comparison with plants subjected to Cd stress without SA application. A statistically-significant increase in Ca, Mg and Fe concentrations (34.9%, 16.3% and 34.9%, respectively) was found only at 100 μM SA as compared to Cd-stressed plants without SA application.

3.4. Effects of SA and Cd on pigment concentrations

The effects of SA and Cd on chlorophyll (Chl) and carotenoid (Cars) concentrations are summarized in Table 5. A significant decrease in concentrations of all pigments under Cd stress conditions was observed. The reductions in Chl*a*, Chl*b*, Chl (Chl*a* + Chl*b*) and Cars concentrations in Cd-exposed plants as compared to the control were 27.3%, 16.7%, 25.4% and 15.6%, respectively. Cd treatment affected equally Chl*a* and Chl*b*; therefore Chl*a*/Chl*b* ratio did not change under Cd treatment. By contrast, the Cd-induced decline of Chl*a* + Chl*b* was more pronounced than that of Cars, thereby leading to an increase in Cars/Chl ratio in Cd-stressed plants (Table 5). Pretreatment with SA did not change pigment concentrations in control plants, whereas it alleviated Cd-induced pigment loss in plants subjected to Cd stress. Chl*a* and Chl*b* were equally protected by SA application, leading to unchanged Chl*a*/Chl*b* ratio. SA effect was more efficient in restoring chlorophyll concentrations than those of carotenoid, which decreased Cars/Chl ratio.

3.5. Effects of SA pretreatment on H_2O_2, MDA concentration and electrolyte leakage in leaves of bean seedlings under Cd stress

Cadmium addition increased H_2O_2 concentration by nearly 3 times as compared to the control (Table 6). SA alone did not significantly affect H_2O_2 production rate (Table 6). By contrast, its concentration in leaves of seedlings supplied with 10, 50 and 100 μM SA was decreased by respectively 18.5%, 48.1% and 58.5% relative to Cd-stressed plants grown without SA application.

As compared to the control, Cd-treated plants exhibited a higher leaf MDA concentration (Table 6). Contrarily, no major changes were observed in MDA level in the presence of SA alone. Plant pretreatment with SA before Cd application significantly decreased MDA level in a dose-dependent manner, the effect being more pronounced with the highest applied SA concentrations. Thus, the observed growth-promoting effect of SA can be partially related to a

Table 3
Uptake of Cd (μg per plant) in bean plants pretreated with different SA concentrations. Means of n = 5 ± SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test).

Cd applied (μM)	SA treatment			
	0 μM	10 μM	50 μM	100 μM
20	196.4 ± 0.143a	142.4 ± 0.203b	122.5 ± 0.463c	91.7 ± 0.112d

Table 4
Effects of SA supplementation on K, Ca, Mg and Fe content in leaves of Cd-stressed bean seedlings. Means of n = 5 ± SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test).

SA (μM)	Cd in nutrient solution	
	0 (μM)	20 (μM)
K (mg g^{-1} DW)		
0	86.330 ± 0.223a	51.29 ± 0.658c
10	139.40 ± 0.682b	84.11 ± 0.914ad
50	130.61 ± 0.031b	77.96 ± 0.524ad
100	119.63 ± 0.957b	73.65 ± 0.603ad
Ca (mg g^{-1} DW)		
0	26.81 ± 0.685a	16.97 ± 0.278b
10	30.75 ± 1.098a	17.58 ± 2.096b
50	25.84 ± 1.054a	20.29 ± 1.410b
100	25.01 ± 0.297a	26.07 ± 0.842ac
Mg (mg g^{-1} DW)		
0	55.98 ± 0.091a	31.93 ± 0.759b
10	45.53 ± 0.010a	39.81 ± 0.727bc
50	47.20 ± 0.779a	34.58 ± 0.513bc
100	47.13 ± 0.897a	44.58 ± 0.062c
Fe (mg g^{-1} DW)		
0	0.98 ± 0.028a	0.49 ± 0.021c
10	1.01 ± 0.112a	0.60 ± 0.020c
50	1.07 ± 0.044a	0.47 ± 0.029c
100	1.55 ± 0.130b	0.89 ± 0.126d

Table 5
Effects of SA pretreatment on chlorophyll and carotenoid content (mg g^{-1} FW) in leaves of Cd-stressed bean seedlings. Abbreviations: Chl, chlorophyll and Cars, carotenoids. Means of n = 5 ± SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test).

SA (μM)	Cd in nutrient solution	
	0 (μM)	20 (μM)
Chla		
0	4.56 ± 0.108a	1.49 ± 0.150b
10	4.48 ± 0.052a	2.83 ± 0.251c
50	4.03 ± 0.166a	2.55 ± 0.081c
100	4.10 ± 0.057a	2.82 ± 0.317c
Chlb		
0	8.29 ± 0.090a	2.76 ± 0.202b
10	7.61 ± 0.401a	5.60 ± 0.464c
50	7.61 ± 0.125a	6.02 ± 0.089c
100	7.71 ± 0.086a	5.72 ± 0.237c
Chl (Chla + Chlb)		
0	12.86 ± 0.080a	4.26 ± 0.162b
10	12.09 ± 0.102a	8.43 ± 0.343c
50	11.64 ± 0.150a	8.57 ± 0.096c
100	11.81 ± 0.093a	8.54 ± 0.164c
Cars		
0	4.02 ± 0.096a	1.87 ± 0.160b
10	3.91 ± 0.087a	2.50 ± 0.097c
50	3.82 ± 0.176 a	2.46 ± 0.065c
100	3.96 ± 0.072a	2.52 ± 0.134c
Chla/Chlb ratio		
0	0.55 ± 0.080a	0.54 ± 0.120a
10	0.59 ± 0.234a	0.51 ± 0.302a
50	0.53 ± 0.145a	0.42 ± 0.050a
100	0.53 ± 0.078a	0.49 ± 0.272a
Cars/Chl		
0	0.312 ± 0.075a	0.441 ± 0.158b
10	0.323 ± 0.153a	0.296 ± 0.082a
50	0.328 ± 0.281a	0.286 ± 0.047a
100	0.334 ± 0.196a	0.296 ± 0.140a

Table 6
Effects of SA pretreatment on H_2O_2, MDA concentration and electrolyte leakage in 14-d-old bean plants submitted for 3 days to Cd stress. Means of n = 5 ± SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test).

SA (μM)	Cd in nutrient solution	
	0 (μM)	20 (μM)
H_2O_2 (nmol g^{-1} FW)		
0	49.23 ± 0.984ae	157.30 ± 0.783b
10	44.08 ± 0.977ac	128.10 ± 0.344c
50	31.73 ± 0.043a	82.83 ± 0.524de
100	34.27 ± 0.756a	65.17 ± 0.875e
MDA (nmol g^{-1} FW)		
0	37.42 ± 0.480a	115.25 ± 0.819b
10	35.04 ± 0.188a	55.53 ± 0.501c
50	28.21 ± 0.833a	48.11 ± 0.962ac
100	37.56 ± 0.933a	21.68 ± 0.389ac
Electrolyte leakage (%)		
0	20.96 ± 0.801a	47.97 ± 0.791b
10	24.00 ± 0.372a	33.06 ± 0.299c
50	24.10 ± 0.101a	35.37 ± 0.145c
100	23.87 ± 0.331a	34.98 ± 0.095c

lower degree of membrane damage as demonstrated by the diminished lipid peroxidation. Electrolyte leakage was also affected by Cd (the increase was approximately 56% compared to the control) and its effect was alleviated by pretreatment with SA (Table 6).

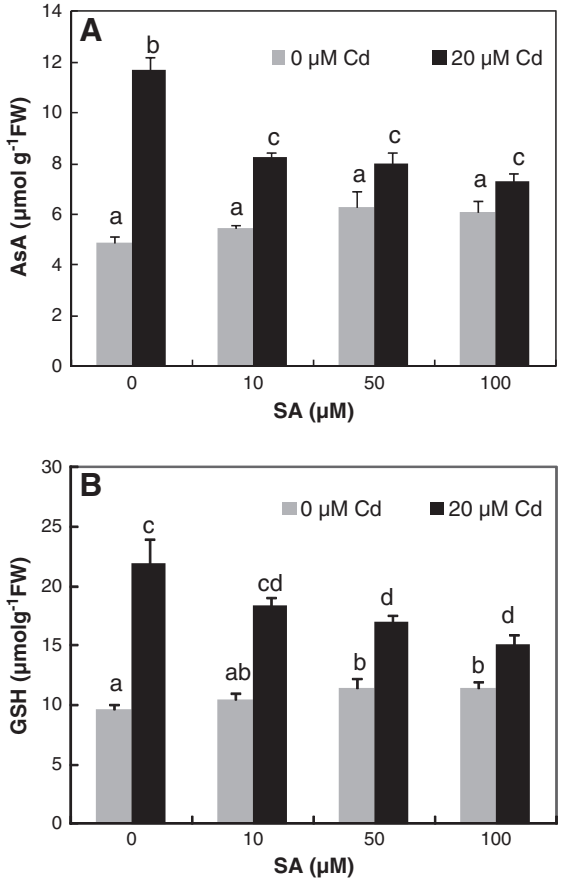

Fig. 1. Effects of SA pretreatment and/or 3-day Cd treatment (20 μM) on leaf AsA (A) and GSH (B) concentrations in bean seedlings. Means of n = 5 ± SE from three independent experiments. Different letters mean significance of difference between the treatments (P<0.05, Tukey's test).

3.6. Effects of SA pretreatment on ascorbate (AsA) and glutathione (GSH) pool in leaves of bean seedlings under Cd stress

As shown in Fig. 1A, AsA concentration increased under cadmium stress conditions by 120%. Such an increase was obviously less pronounced in SA-pretreated plants before Cd application. In plants pretreated with SA and non-subjected to Cd stress, no significant difference with the control was detected.

Leaf GSH concentration significantly increased (+56.0%) under Cd stress conditions as compared to control (Fig. 1B). This increase was reduced by SA pretreatment by 22.5% and 31.5%, respectively at 50 and 100 μM. By contrast, in the absence of Cd, SA application at 50 and 100 μM significantly increased leaf GSH concentration.

3.7. Effects of SA pretreatment on antioxidant enzyme activities in leaves of bean seedlings under Cd stress

SOD and POD activities in leaves exposed to 20 μM Cd were observed to be 56–57% higher than those of the control (Fig. 2A–B). By contrast, CAT and APX activities were decreased after exposure to Cd; they were about 50% of the control (Fig. 2C–D). SA pretreatment resulted in a significant decrease in SOD and POD activities upon Cd exposure and alleviated the inhibitory effect of Cd on CAT and APX activities. The most prominent effect was at 100 μM SA, the concentration that induced an increase of 33–38% in CAT and APX activities (Fig. 2C–D). However, no noticeable variation was observed in antioxidant enzyme activities of plants treated with SA only, except the significant increase in CAT and POD activities at 100 μM SA.

4. Discussion

In the present study, short-term Cd stress caused drastic reduction of bean seedling growth, which could be due to the inhibition in cell division and elongation rate that mainly occur by an irreversible inhibition of proton pump responsible for the process (Fodor et al., 1995). Pretreatment with SA before Cd exposure resulted in significant increase in root and shoot biomass, when compared to Cd-stressed plants grown without SA addition. The ameliorative impact of SA on growth as observed in the present study has already been reported in different crop plants under abiotic stress conditions and this was ascribed to the role of SA in Cd distribution (Belkhadi et al., 2010), nutrient uptake (Glass, 1974), water relations (Barkosky and Einhellig, 1993), stomata regulation, photosynthetic capacity and growth rate (Arfan et al., 2007).

Cadmium is distributed in bean seedlings mainly in roots and to a lesser degree in leaves. Such metal immobilization in root cells is related to an exclusion strategy (Baker et al., 2006). A moderate resistance to heavy metals can be realized by selective Cd exclusion and/or lowered uptake by mechanisms, leading to lower cytoplasmic Cd contents (Hall, 2002). Our data indicated that SA significantly decreased Cd uptake and translocation to shoots in a dose-dependent manner. The SA-induced differential accumulation of Cd was considered as one of the important causes for SA-induced Cd tolerance in bean plants.

Variations in K, Ca, Mg and Fe concentrations indicate that Cd significantly disturbs ionic homeostasis and that SA stimulated their maintenance in bean leaves. SA-induced increase in leaf K, Ca, Mg and Fe concentrations may be due to increased ion uptake and/or increased ion transport into shoots. SA-induced H^+-ATPase activity (Gordon et al., 2004) might be responsible for the increase in Ca and Mg absorption under Cd stress since this pump is involved in plasma membrane ion transport (Palmgren and Harper, 1999). Moreover, SA as a signaling molecule can change plasma membrane properties and affect ion channel activity (Engelberth et al., 2001). This could be responsible for the nutrient homeostasis of bean under excess Cd.

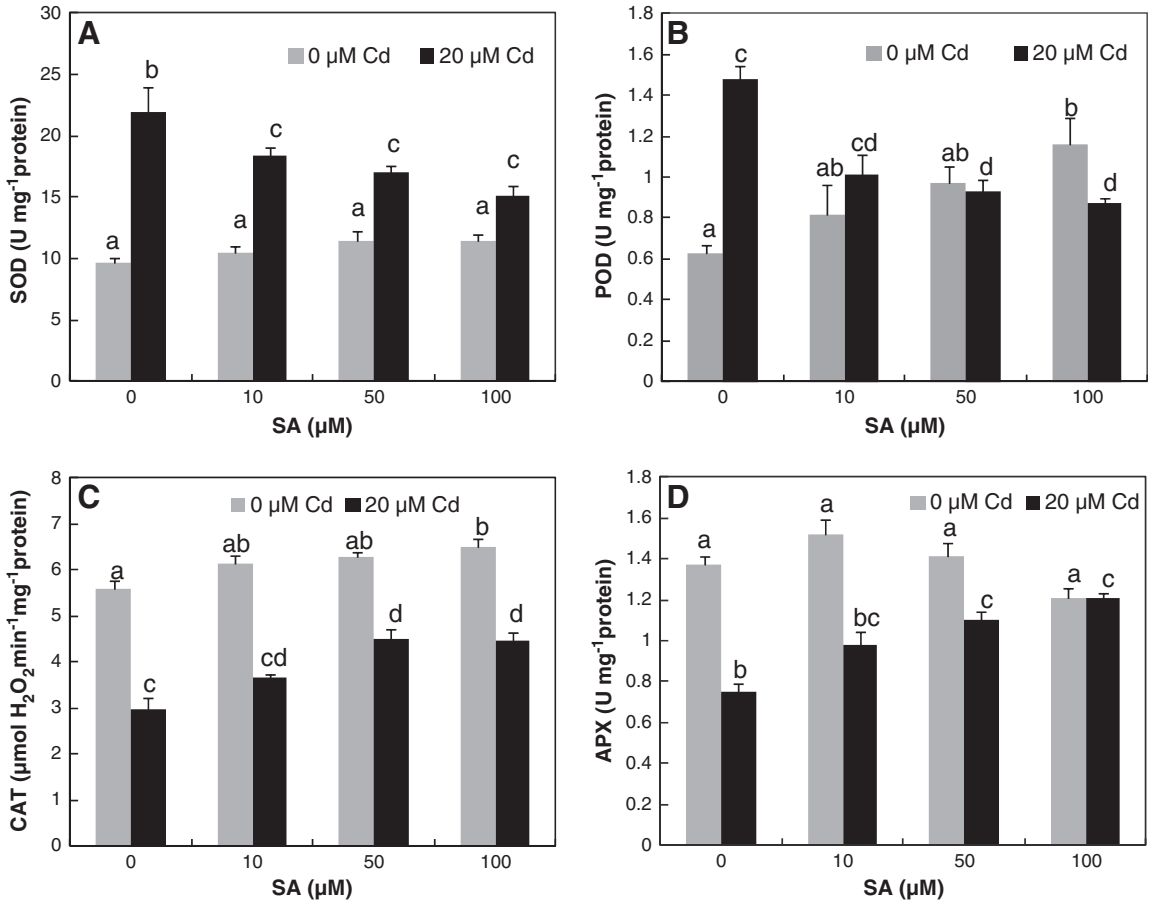

Fig. 2. Effects of SA pretreatment and/or 3-day Cd treatment (20 μM) on leaf SOD (A), POD (B), CAT (C) and APX (D) activities in bean seedlings. Means of n = 5 ± SE from three independent experiments. Different letters mean significance of difference between the treatments ($P<0.05$, Tukey's test).

In the present study, SA enhanced the accumulation of both chlorophylls and carotenoids in leaves under Cd stress. The obtained results are in agreement with the findings of Belkhadi et al. (2010) who reported that seed priming with SA increased chlorophyll and carotenoid concentrations in flax leaves. This might be attributed to an efficient scavenging of ROS by antioxidant enzymes or, otherwise, they would have destroyed photosynthetic pigments. Increasing carotenoid level can protect the photosynthetic membrane against photo-oxidation by quenching the triplet states of chlorophyll molecules as well as effectively scavenging ROS, protecting pigments and unsaturated fatty acids of lipids from oxidative damage (Strzałka et al., 2003).

Short-term Cd stress resulted in increased activities of SOD and POD. Simultaneously, a decrease in CAT and APX activities was observed and was possibly due to the binding of Cd to the thiol groups of enzyme proteins (Van Assche and Clijsters, 1990). Consequently, the high homeostatic balance of ROS was probably broken, causing a H_2O_2 burst after 3 days of Cd treatment. Pretreatment of bean seedlings for 7 d with SA before exposure to Cd led to a decrease in oxidative injuries as evidenced by decreased H_2O_2 and lipid peroxidation levels. SA influence on avoiding the toxic effects of Cd may be a consequence of very different primary effects connected with oxidative stress (Borsani et al., 2001) and stabilization of cell membranes (Mishra and Choudhuri, 1999), leading to the increase of general stress tolerance. SA may act directly as an antioxidant to scavenge the ROS and/or indirectly modulate redox balance through activation of antioxidant responses. Indeed, it is a direct scavenger of hydroxyl radical and an iron chelating compound, thereby inhibiting the direct impact of hydroxyl radicals as well as their generation via the Fenton reaction (Dinis et al., 1994).

In SA-pretreated bean plants, the initially decreased activities of SOD and increased activities of CAT and APX cooperatively controlled the Cd-induced H_2O_2 at high homeostatic levels contrarily to the mode during plant–pathogen interactions (Chen et al., 1993). It seems to suggest that SA-reduced H_2O_2 permit bean plants to respond more effectively to Cd-induced oxidative damage. It is well established that CAT has a high reaction rate but a low affinity for H_2O_2, whereas APX has a high affinity for H_2O_2 and is able to detoxify low concentrations of H_2O_2 (Mittler, 2002). Therefore, it is possible that stimulation of CAT and APX activities by SA decreases the level of H_2O_2 in bean leaves, which may be a possible mechanism in plant defense strategy against Cd-induced oxidative stress.

In bean leaves, Cd stress led to increased AsA and GSH levels. It is conceivable that adaptations of intracellular AsA and GSH to Cd might clearly depend on the balance between the rates and capacities of their biosynthesis and turnover related to antioxidant demand. By contrast, SA pretreatment was shown to decrease the concentration of AsA and GSH in leaves of bean seedlings under Cd stress. This could reflect an intensive use of these metabolites as consequence of SA pretreatment. The protective role of SA in Cd-stressed plants can be attributed to (i) SA-regulated Cd uptake, transport and distribution in plant organs, leading to decrease in Cd concentration in leaf tissues, (ii) SA-inhibited lipid peroxidation process, contributing to membrane stability and (iii) SA-controlled Cd-induced H_2O_2 at high homeostatic levels by modulating enzymatic (SOD, APX and CAT) activity. SA might influence H_2O_2 signaling pathways in plant defense against Cd,

should be further investigated to dissect the complicated network of SA and its involvement in plant defense at a molecular level.

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